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REVIEW

Hormonal Influences on the Development of Amphibian Pigmentation Patterns*

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ABSTRACT—The Mechanisms responsible for pigment pattern formation in amphibians and other vertebrates are little understood, although it is known that a fundamental process is the migration of uncommitted chromatoblasts from the neural crest to the integument. Here, in response to appropriate cues present in the integumental milieu, the various pigment patterns such as dorsal/ventral, spots, stripes, etc., are expressed. Because of its importance in color change, melanocyte stimulating hormone (MSH) has been suggested to play a role in pigment pattern formation, but current knowledge suggests that it functions only as an enhancer or modifier of already determined patterns through the recruitment of latent melanoblasts or the stimulation of melanophore proliferation. Similarly, the profound changes in pigment pattern at metamorphosis result from the permissive action of thyroxine that allows for the expression of already pre-determined pigmentation patterns. Sex steroids may also play a role in pigmentation pattern changes during sexual maturation. At present, the most promising candidates as causal agents in the determination of pigmentation patterns are some large protein molecules present in the skin where they exert their effects in situ. These include a melanization inhibiting factor (MIF), prevalent in the ventral skin and presumably responsible for the pale ventrum of frogs and a melanization stimulating factor (MSF), most prevalent in dark dorsal skin and especially in dark spots of the dorsum. A possible interaction between MIF and MSF and an interplay of these factors with hormones such as MSH and thyroxine may very much influence the expression of pigmentation patterns.

INTRODUCTION

Amphibians have long been the target of studies on pigmentation and since the beginning of the century investigations have largely centered on endocrine control of color change and the development of pigmentation patterns [5, 10]. Knowledge gained from such studies has been extended to other vertebrates and amphibians have continued to provide a model system in continuing investigations. Great progress has been made in the understanding of the physiology of color change, but by comparison, the elucidation of mechanisms responsible for the development of pigmentation patterns has lagged behind. Fortunately, new knowledge in this area has emerged from more recent studies on amphibians and it offers the promise of explaining the developmental origins of vertebrate pigmentation in general. It is the aim of this presentation to summarize our current understanding of the mechanisms that provide the basis for pigment pattern formation in amphibians.

Of fundamental importance is the fact that the seemingly different pigment cell types typical of amphibians, although different in form, function and composition, are in reality very much closely related to one another [13]. A key to this relationship resides in their common neural crest ori-

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* Dedicated to the memory of Professor Johannes F.C. Holtfreter, an inspiring undergraduate teacher of J.T.B. and a long time friend of Japan.

gin [14]. Thus, the epidermal melanocyte(-phore), with its elongate bipolar form and the large and broad dermal chromatophores, including melanophores, iridophores and xanthophores are all derived from the neural crest and their constituent pigment-containing organelles originate from a primordial vesicle derived from the rough endoplasmic reticulum. According to current thinking, the various pro-pigment cells or chromatoblasts, which ultimately migrate over the entire surface of the tailbud embryo to provide the pigment cells of the adult integument and other peripheral tissues, are largely undetermined when they commence their migration, but during the migratory process, or at some point after they reach their ultimate destination, their developmental fate is determined (for a more complete discussion, see [8, 9]). The present discussion is aimed toward understanding what factors impinge upon chromatoblasts causing them to differentiate in one direction or another as pigment patterns develop. While there is some evidence that extracellular matrix molecules play a role in chromatophore differentiation of salamander chromatoblasts during the migratory phase [20], two other major foci are primarily addressed herein. One focus is a hormonal one featuring the action of melanocyte stimulating hormone (MSH) and thyroxine, under whose aegis profound changes in pigment cell expression and pattern formation occur at metamorphic climax. Another is *in situ* protein factors of the integument that seem to be particularly important in the expression of pigmentation patterns. The action of hormones in this regard is an older theme broadly covered in previous reviews [9, 10], while intrinsic pigmentary factors of the integument is a newer concept that is just emerging [15]. The later concept owes its origins to the work of Fukuzawa and Ide [26] who discovered the presence of a melanization inhibitory factor (MIF) whose restricted presence in the ventral surface of *Xenopus* was taken to provide the basis for the dorsal/ventral pigmentation of that species.

CELLULAR BASIS FOR PIGMENTATION PATTERNS

Amphibians and other cold-blooded vertebrates are rich in their variety of pigmentary patterns that include spots, stripes and mottlings of various colors. These circumscribed patterns are achieved by an appropriate distribution of the various chromatophore types, in particular, the dermal chromatophores. It is beyond the purpose of this paper to review the nature of amphibian pigment cells, for such has been documented in detail [5]. Nevertheless, it should be pointed out that the fundamental pigment cells of amphibians are the dermal chromatophores that include melanophores, xanthophores (erythrophores), and iridophores. Often these chromatophores are active in bringing about color change due to the intracellular migration of their respective pigmentary organelles in response to various stimuli, usually hormonal (physiological color change).

Melanophores are the most well known of the dermal chromatophores and these broad and dendritic pigment cells contain eumelanin in organelles called melanosomes. Melanosomes disperse to the periphery of the dendrites in response to MSH and thus, melanophores are of key importance during integumental darkening. Melanophores are also found in the epidermis in specific localities during larval and/or adult life. These epidermal pigment cells are like the epidermal melanocytes of all other vertebrates and are of special importance to birds and mammals wherein they serve as agents of morphological color change through the deposition of cytocrine packets of melanosomes into keratinocytes, hair, or feathers. While epidermal melanocytes respond to MSH by dispersing melanosomes, they are of relatively little importance in physiological change because of their thin spindle shaped form which can cover only a minimal surface area. Generally, there is a negative correlation between the distribution of dermal chromatophores and epidermal melanocytes such that in areas of the skin having well formed dermal chromatophore units, epidermal melanocytes are fewer in number and vice versa [12]. This makes sense since the presence of static deposits of epidermal melanin would obscure color changes brought about by organellar movements within dermal chromatophores beneath.

Iridophores are primarily involved in the production of structural colors due to the highly

organized deposition of crystalline pigments in organelles termed reflecting platelets. Like melanophores, they are very active in physiological color change and are also regulated by MSH [2]. Since they most often reflect all wavelengths of light, they appear whitish under surface illumination and thus, in the absence of MSH, they contribute to the whiteness of the dorsal surface. However, when animals darken under MSH stimulation, iridophores aggregate their reflecting platelets and the color of the animal (frog) is dominated by melanophores whose pigment-filled dendrites cover a large surface area. Iridophores, relatively insensitive to MSH, dominate the ventral surface of most frogs and other amphibians.

Xanthophores are yellowish pigment cells that can approach orange or red (erythrophores) and often work together with underlying iridophores to provide yellow, orange or red areas. While these chromatophores are also active in physiological color change [4], this role is less clearly visible on a cellular basis. A pronounced function of xanthophores is in the production of green coloration wherein yellow xanthophores work together with deeper lying iridophores (which produce blue color through light scatter) and melanophores to provide a dermal chromatophore unit [12].

HORMONES AND THE DEVELOPMENT OF PIGMENTATION

The Role of MSH

The possibility that MSH may play a major role in the developmental biology of pigment cells has long been an attractive one. First evidence in this regard stemmed from the observation that *Xenopus* larvae, hypophysectomized as embryos, possess many fewer melanophores than do their intact siblings [1]. Moreover, such larvae possess iridophores in areas of the integument where only melanophores normally occur. It was concluded that MSH had a trophic influence that led to the differentiation of melanophores and at the same time, inhibited the differentiation of latent iridophores (iridoblasts). Similarly, Hadley and Quevedo [29] had demonstrated that adult *Xenopus* kept on dark backgrounds for 10 weeks possessed many more integumental melanophores than did toads kept on white backgrounds for the same period. Moreover, when returned to a white background, the darkened individuals began to lose some of the supernumerary melanophores. Presumably, the respective presence or absence of circulating MSH was responsible for these effects on melanophore number. While it seemed implicit from both of these studies that the increase in melanophore number resulted from the recruitment of latent melanoblasts present in the skin, the possible role of MSH on melanophore proliferation could not be excluded. Indeed, in some elegant experiments, it was shown conclusively by Pehlemann [38] that inordinantly high levels of circulating MSH in *Xenopus* larvae leads to profound proliferation of existing melanophores and subsequent darkening of the integument. Despite the unequivocal demonstration that MSH induces melanophore proliferation, the question of its possible role in stimulating the differentiation of latent melanoblasts still remained unanswered. This, too, was resolved by some experiments (Bagnara, unpublished) wherein it was found that following the homoplastic implantation of a pars intermedia in the normally unpigmented lower jaw of *Xenopus* larvae, dermal melanophores were expressed in a concentric radiating pattern from the implant (Fig. 1). Obviously, MSH diffusing in an uncontrolled manner from the pars intermedia induced melanization of latent neural crest cells that were present in the skin of the lower jaw. Thus, it seems clear that MSH can exert trophic effects, based upon both proliferation and cellular differentiation, in larvae and adults.

The results described so far, are for the most part from experiments wherein profound levels of MSH secretion were altered by appropriate surgical procedures and the question arises of whether these are extreme situations that may not occur in nature. Actually, there is good evidence that natural fluctuations in circulating MSH levels can have a profound effect on the development of pigment cells and the expression of pigmentation pattern. One such study is that of Fernandez and Collins [22] who showed that the environmental background during ontogeny can profoundly influence the expression of the color pattern of

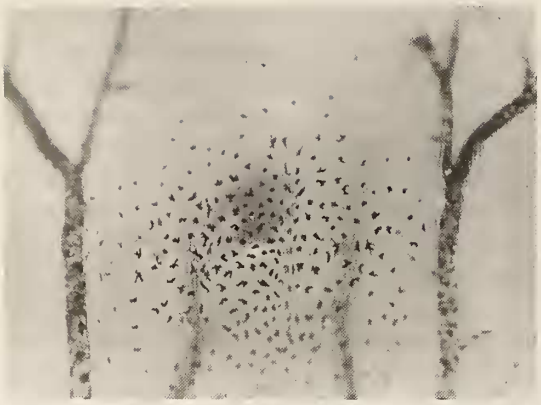


Fig. 1. Lower jaw region of a larva of *Xenopus laevis* that had been implanted subcutaneously with the pars intermedia of a froglet. A spherical blood clot marks the site of the transplant which releases MSH chronically. Note that the nearby dermis is populated with melanophores distributed over the graft. No melanophores were observed in the dermis of the jaw before the transplantation.

Arizona tiger salamanders (*Ambystoma tigrinum nebulosum*) at metamorphosis (Fig. 2). In turbid ponds, larvae are exposed to a high albedo and such larvae are very lightly colored presumably due to diminished circulating levels of MSH. In contrast, genetically related populations in clear ponds reside in low albedo environments that stem from the absorption of light by the dark pond bottom. Such larvae are darkly colored presumably due to the elevated levels of MSH that are present during larval development. That these color differences actually resulted from appropriate variations in MSH levels was supported by the fact that animals from clear ponds had many fully expanded melanophores while those from the high albedo turbid ponds possessed four times as much integumental guanine, the primary constituent of iridophore reflecting platelets, than the low albedo dark colored larvae [21].

An even more striking example of the effects of MSH on the morphological expression of pigmentation pattern is manifested in the leopard frog, *Rana chiricahuensis* (Fig. 3). Unlike other sibling species of leopard frogs (Fig. 4) which have a markedly pale ventrum in contrast to a much darker dorsum, *R. chiricahuensis* has the capacity to express a heavily melanized ventral surface [6]. The comparative ontogeny of ventral pigmentation in the more melanistic species, *R. chiricahuensis* and the pale species, *R. pipiens* has been studied [24]. It was found that while the ventral skin of larvae of both species is phenotypically similar in that only iridophores are present (Fig. 5A, E), marked differences exist in newly metamorphosed frogs (Fig. 5B, F) in that melanophores are already present, along with iridophores, in *R. chiricahuensis,* but none ever develop in *R. pipiens* whose ventral surface is populated exclusively by iridophores. The number of ventral melanophores has increased by the adult stage of *R. chiricahuensis* and these are capable of physiological color change on dark and light backgrounds (Fig. 5C, D, G, H). The question of why *R. chiricahuensis* exhibits ventral melanization wherein this closely related sibling species [31] does not, can not be fully answered at this time, but there is good reason to conclude that the explanation involves MSH action. It has been shown that *R. chiricahunensis* has the capacity to secrete extraordinary amounts of MSH (Fig. 6), such that upon black background adaptation, plasma MSH levels can be eight times greater than those of *R. pipiens* [23]. Similarly, low temperatures (5°C) induce the same high plasma MSH levels in *R. chiricahuensis,* but not in *R. pipiens.* Therefore, it would seem that *R. chiricahuensis,* a species that thrives in elevations as high as 9,000 fit, is chronically exposed to lower temperatures and thus would be expected to possess relatively high plasma levels of MSH throughout most of the year. Accordingly, it seems likely that the presence of ventral melanization might be due to the recruitment of latent melanoblasts by MSH, just as was suggested for *Xenopus* adults under long term black background adaptation. It would be possible to speculate about other explanations for the ventral melanization of *R. chiricahuensis*; however, it seems most probable that this darkening results from chronically elevated levels of MSH.

The examples presented so far about possible stimulatory effects of MSH on melanophore differentiation, pertain to relatively late stages in development while, in fact, chromatoblasts have long since completed their migrations by this time and embryonic and larval pigmentation patterns

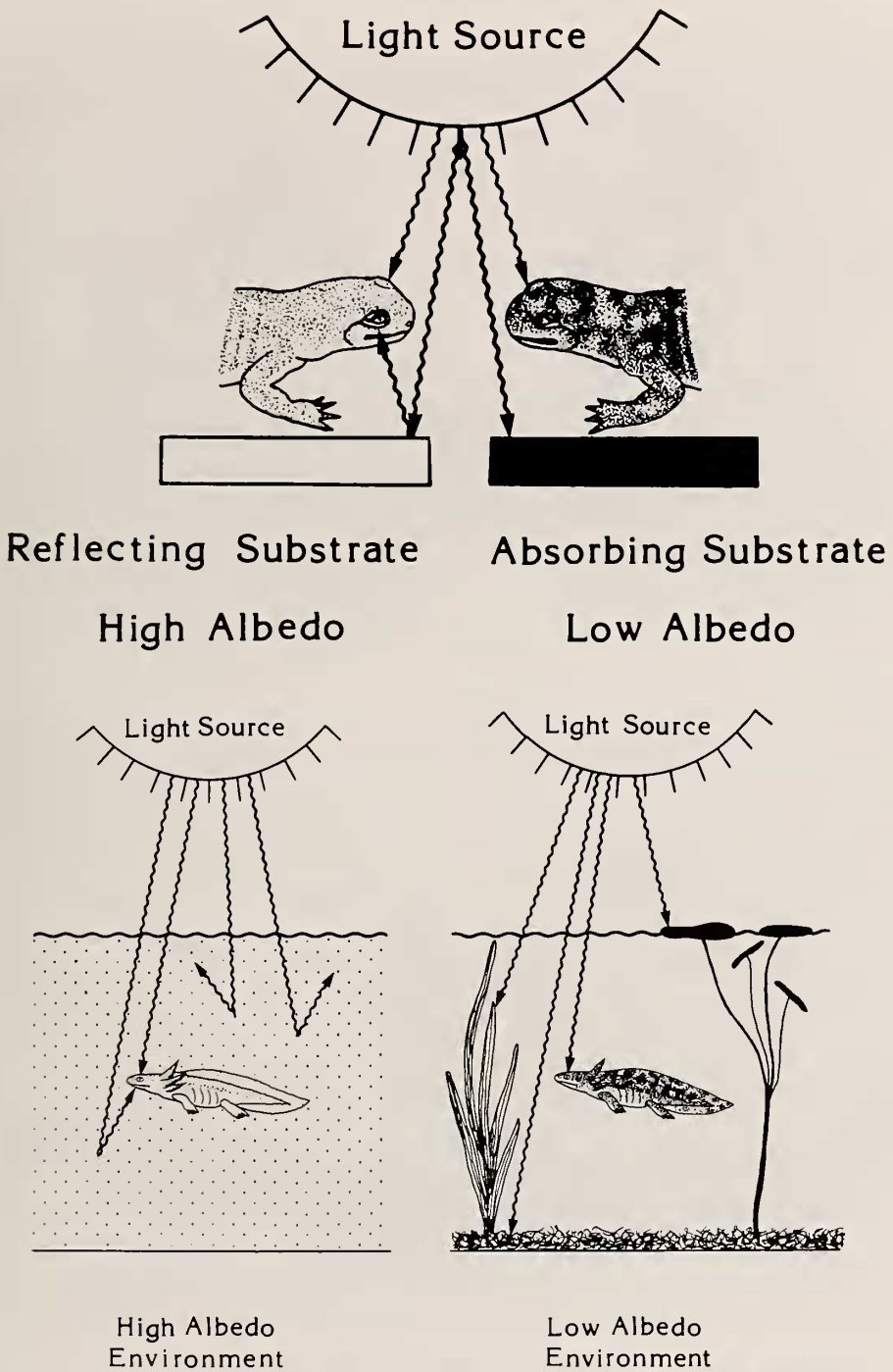


FIG. 2. Perception of light by Arizona tiger salamanders differs between white and black substrates. Light is reflected from a white substrate and provides a high albedo whereas a black substrate absorbs light and the albedo is low. Larvae in a turbid pond (lower left) experience a high albedo because of light reflected from suspended particles while those in a clear pond are subjected to a low albedo because light is absorbed by plants and the dark-colored pond bottom. (From [22]).

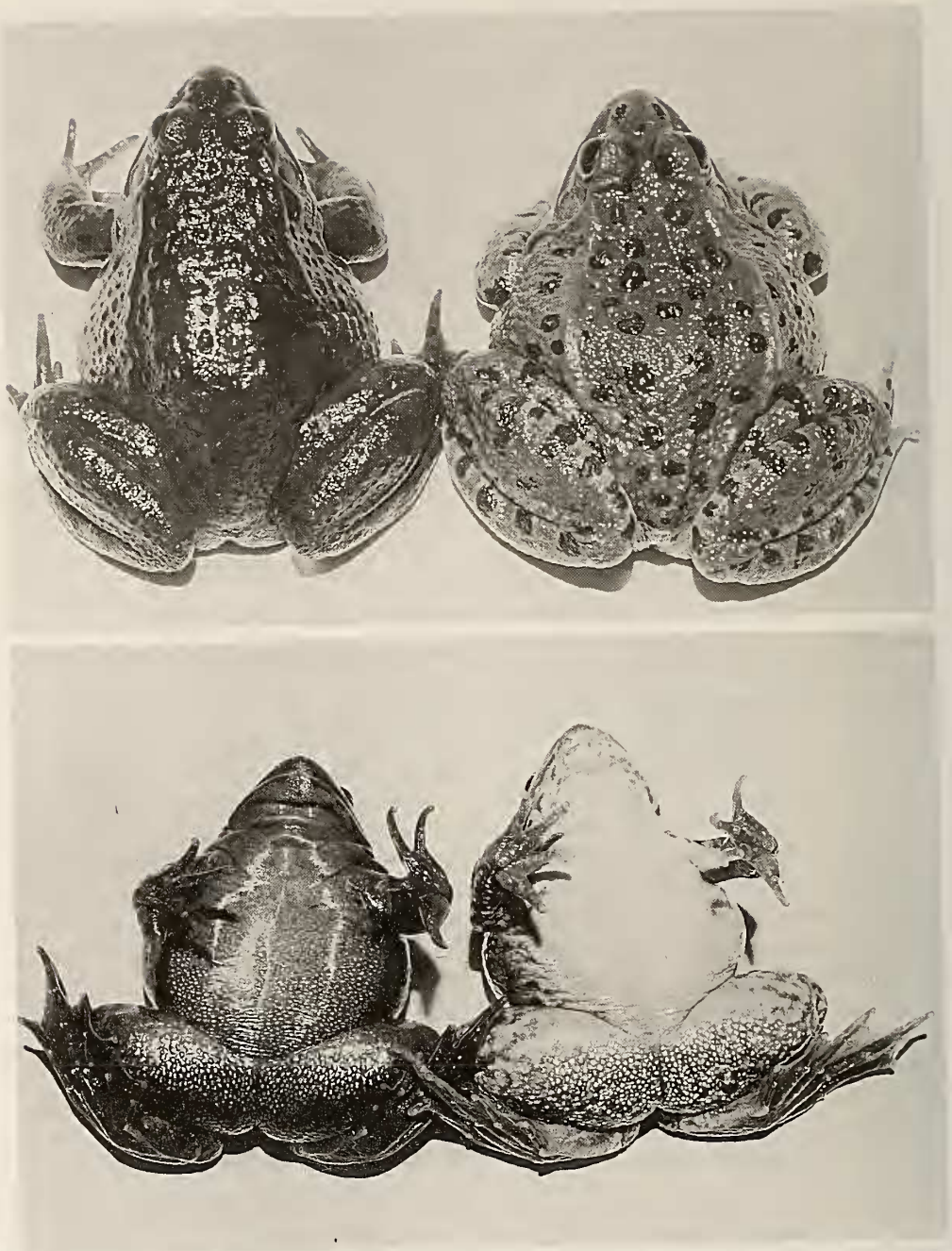


Fig. 3. Dorsal (upper) and ventral (lower) views of adult *R. chiricahuensis* adapted to dark backgrounds (left individuals) or white backgrounds (right individuals). Note the profound darkening of the dark background adapted individuals and especially of the ventral surface.

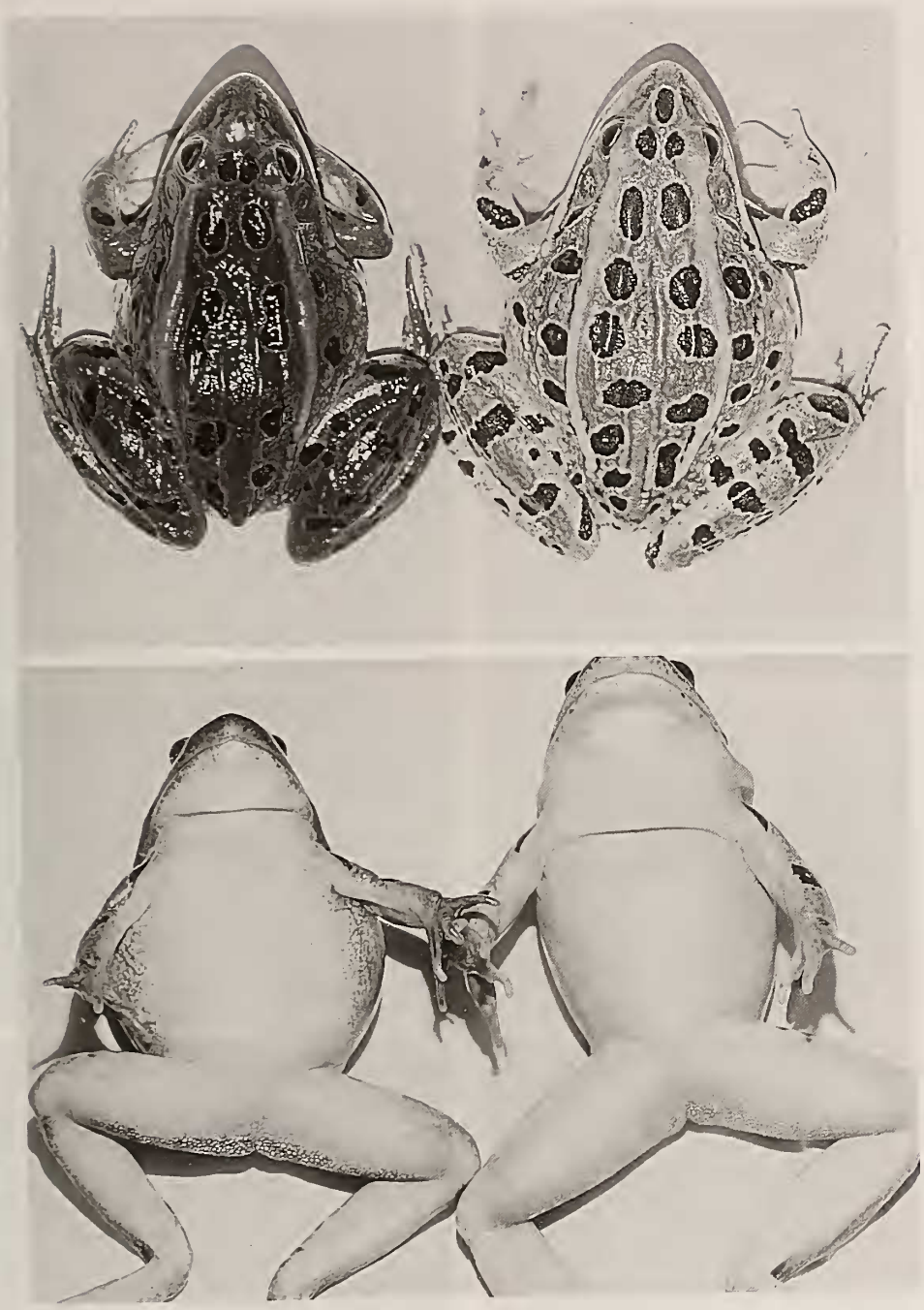


FIG. 4. Dorsal (upper) and ventral (lower) views of adult *R. pipiens* adapted to dark backgrounds (left individuals) or white backgrounds (right individuals). Note the darkening of the dorsal surface of the dark background adapted individuals and the lack of darkening of the ventral surface of the dark background, adapted individuals.

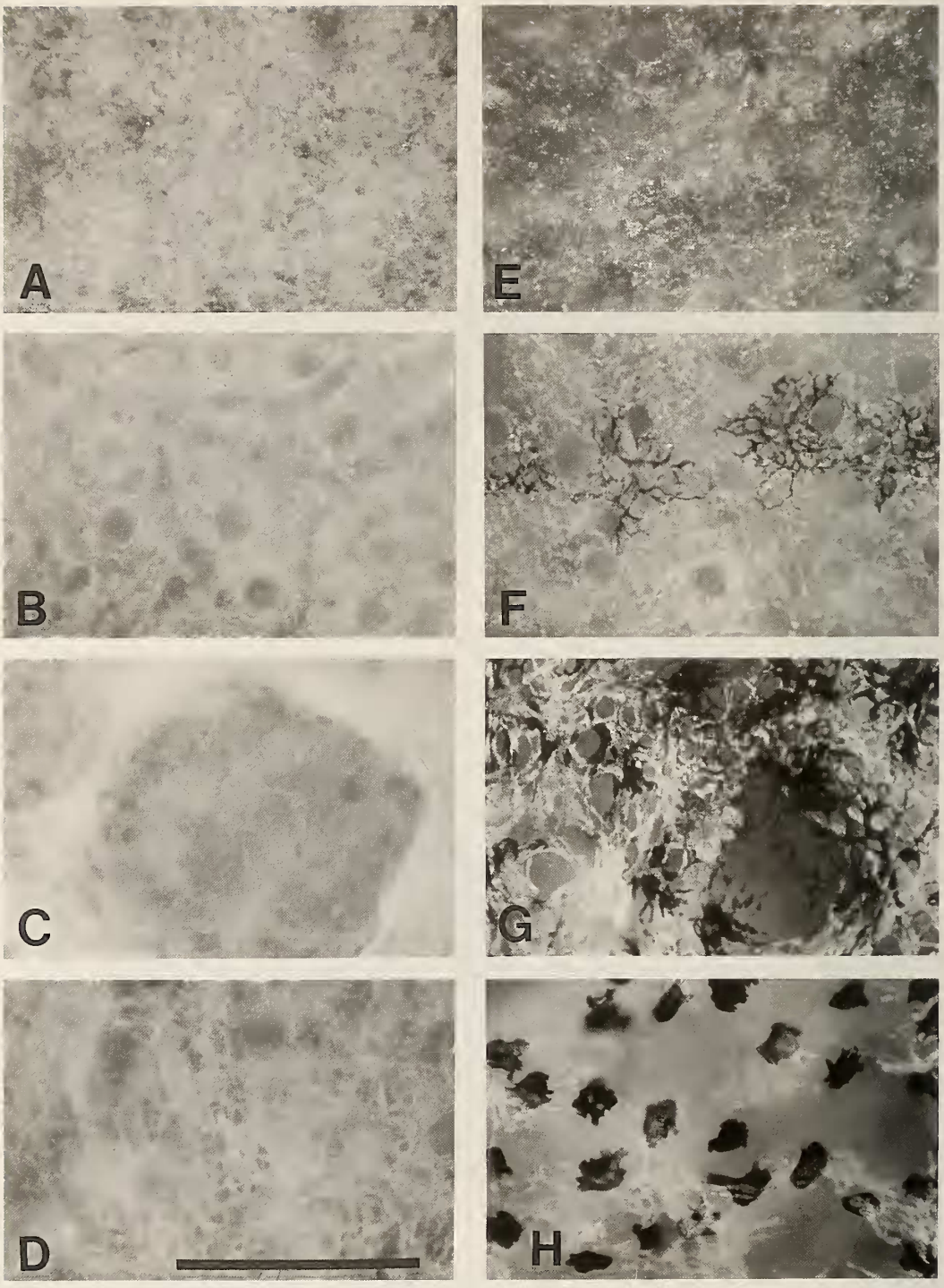


Fig. 5. Whole mounts of *R. pipiens* (A-D) and *R. chiricahuensis* (E-H) ventral skin photographed with reflected light optics. Skin samples are from tadpoles (A, E), newly metamorphosed frogs (BF), adult frogs from a black background (C, G) and adult frogs from a white background (D, H). Bar=100 μm. (From [24]).

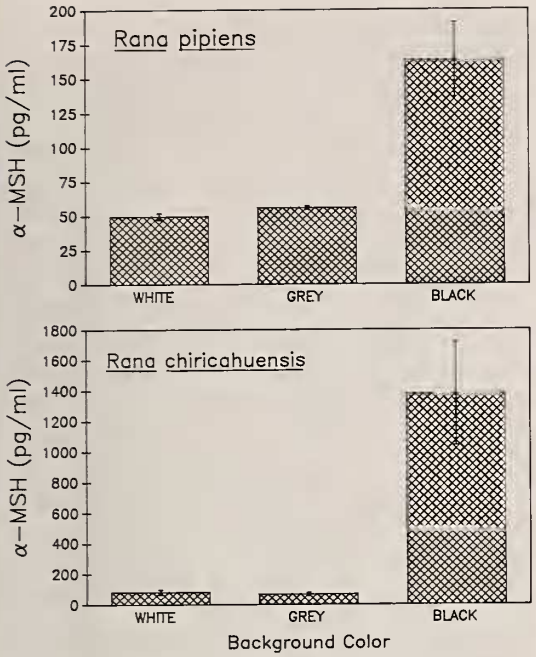


FIG. 6. Plasma α-MSH of *R. pipiens* and *R. chiricahuensis* on white, gray, and black backgrounds. Each bar represents the mean (±SE) of 3–4 frogs. Plasma α-MSH of frogs on a black background is greater than those on white or gray ($P<0.05$). Note the particularly profound increase of plasma α-MSH of *R. chiricahuensis*. (Modified from [23]).

are already expressed [35]. Nevertheless, the possibility that MSH affects early melanophore development has been attractive and the subject of several investigations. It has been known from many years that embryonic salamander melanoblasts are sensitive to MSH [37], but it was not until the experiments of the Wayne State University group that the role of MSH on melanophore development was addressed directly [45, 46]. They found that the exposure of whole *Xenopus* and *Pleurodeles* embryos to MSH or cyclic AMP during the first few days of embryonic development slightly accelerated melanophore differentiation, but that within two or three days, the number of differentiated melanophores on control or treated embryos was the same. They concluded that MSH does not play a role in the normal development of embryonic melanophores.

At this point, we are left with the conclusion that the effects of MSH on chromatophore development are limited to larval, post-metamorphic and adult stages. Since the transition from larval to adult pigmentation at metamorphosis is so pronounced and is characterized by the expression of the adult pigment pattern of the dorsum, the question arises of a possible MSH role in this process. For example, does MSH play a role in the development of the spot pattern of adult leopard frogs? An important clue to the answer of this question has been available for a long time, but has not been appreciated. In particular, we refer to the result of some endocrine experiments wherein tadpoles of several anuran species were manipulated such that they were deprived of a pars intermedia, and thus a source of endogenous MSH, but retained enough pars distalis to induce metamorphosis [18, 19]. Such metamorphosing individuals displayed evidence of a dorsal pigmentation pattern, albeit pale, even in the absence of circulating MSH. A more striking example of this phenomenon resulted from the experiments of Hanaoka [30] who deprived *R. pipiens* tadpoles of a hypothalamus by removing the hypothalamic primordium from the open neural plate stage [16]. The silvery tadpoles that resulted lacked both a hypothalamus and a pars intermedia, but possessed enough of a pars distalis to initiate metamorphosis. These individuals, which remained in premetamorphic stasis, expressed clear spot patterns typical of the adult. A similar tadpole is shown in Figure 7 wherein a silvery tadpole, naturally deprived of a pars intermedia due to a developmental anomaly, reached the start of metamorphic climax and displayed the adult spot pattern (Bagnara, unpublished). These observations show rather conclusively that MSH is not a causative factor in the development of basic pigmentation patterns, but may play a modifying role by stimulating melanophore expression including the recruitment of latent melanoblasts, and inhibiting both the expression of iridophores [2] and the recruitment of latent iridoblasts [1, 9].

A final potential role for MSH in the developmental biology of pigmentation relates to the migration of neural crest cells from the neural tube. Through the use of immuno-fluorescence labeling with an anti-α-MSH polyclonal antibody, Frost-Mason *et al.*, [25] have detected the presence

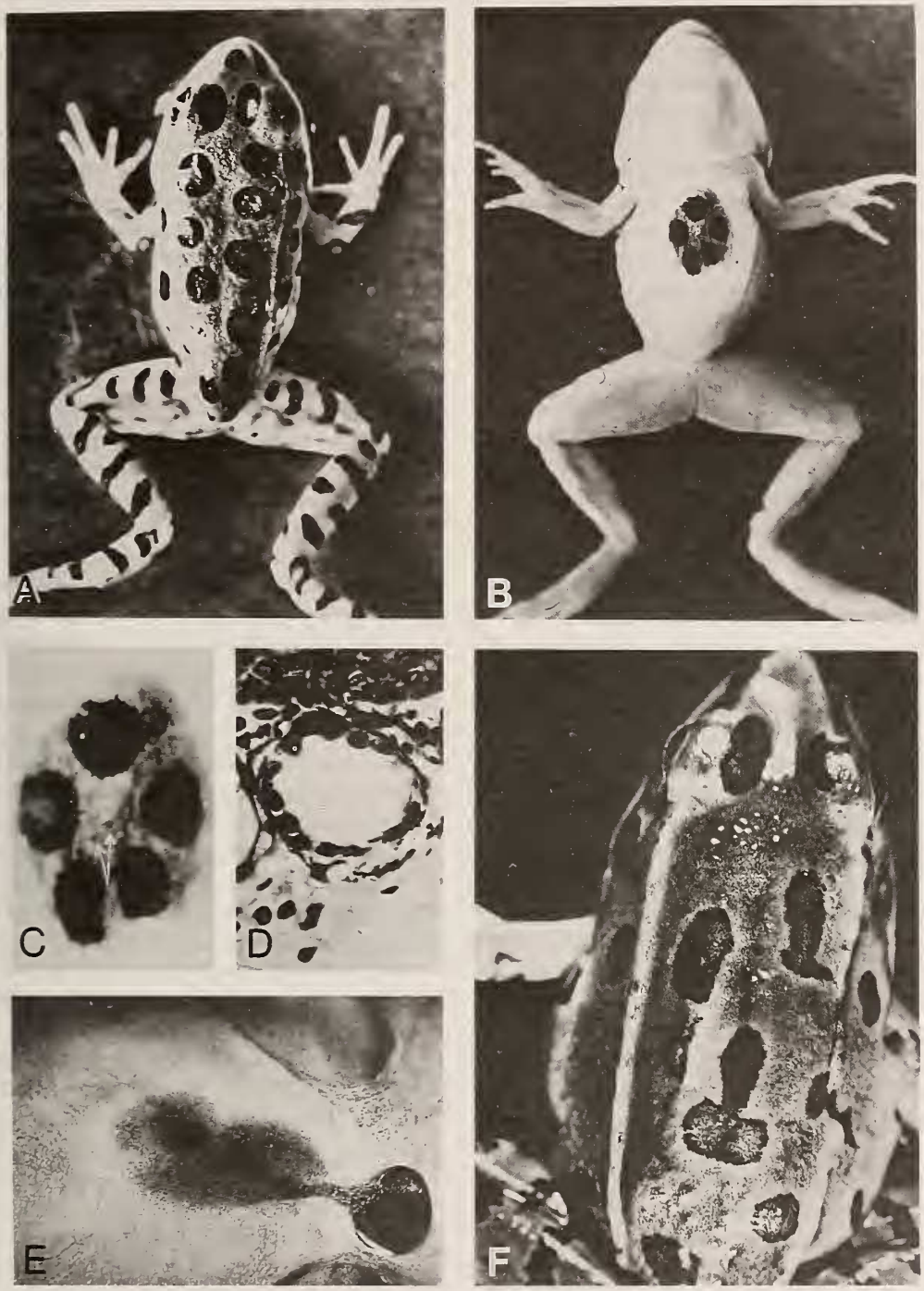
Fig. 7. A *R. pipiens* tadpole in metamorphic stasis due to an anomalous development of the hypophysis such that MSH is lacking and TSH is insufficient to induce metamorphic climax. Note the obvious onset of the formation of a typical spot pattern even in the absence of MSH.

of MSH along surfaces which may be principal neural crest migration routes. The MSH seems to be in association with high molecular weight molecules that may be part of the extracellular matrix of the embryos. It is attractive to speculate that such localizations of MSH are implicated with the pattern of neural crest cell migration or localization and that thus, MSH may play a role in the development of pigmentation patterns. However, research in this area is in its infancy and for this reason together with those mentioned above, it seems premature to conclude that MSH plays a role in pigment pattern formation by regulating chromatoblast migration.

The Role of the Thyroid

Among the myriad of morphological, physiological, and biochemical changes that occur at amphibian metamorphosis is the change from the relatively drab pigmentation of the larva to the striking coloration of the adult. Since practically every event in amphibian metamorphosis is under the direct control of thyroid hormones, notably thyroxine, it follows that the expression of the adult pigmentation pattern is regulated by thyroxine. Indeed, it is a well established fact that localized changes in pigmentation can be induced by local exposure to thyroxine as shown by Kaltenbach [34] who observed the development of adult pigmentation patterns in the vicinity of thyroxine/cholesterol pellets implanted sub-cutaneously in *R. pipiens* tadpoles. In considering these thyroxine mediated changes that occur at metamorphosis, it is important to understand that with respect to pigment pattern formation, the role of thyroxine is a permissive one. The hormone does not determine the pigment pattern that develops; rather, it permits the already pre-programmed pattern to be expressed. While this concept is almost self-evident, clear cut proof derives from simple experiments on tadpoles of *R. pipiens* wherein pieces of dorsal skin were rotated by 90° or by 180° [7]. At this stage, no morphological indications of the future spot pattern are in evidence and the tadpole dorsal integument seems more or less uniformly pigmented in drab brown or black. Nevertheless, a very precise spot pattern is already in place and merely awaits the appropriate metamorphic cue for its expression. This is shown by the fact that in the rotation experiments, prospective spots are often transected such that the unrotated part of a spot remains in place, but is missing a portion which is in the rotated skin piece displaced 90° or 180°, depending upon the degree of skin rotation. At metamorphosis, when the action of thyroxine induces the expression of the adult pigmentation pattern, the effects of the skin rotations are manifested in appropriately disrupted spot patterns (Fig. 8F). Clearly then, thyroxine does not induce the formation of pigmentation pattern, but rather

Fig. 8. A. Dorsal surface of newly transformed froglet of *R. pipiens* showing a large spot pattern. B. Ventral surface of same frog bearing neural fold graft. Note the pattern of small spots. C. Enlargement of graft area demonstrating that it represents an island or dorsality. Small black spots (arrows) are openings of skin glands. Such glands are not found in ventral skin. D. Section through skin gland of the ventral graft. E. Ventral graft of



dorsal ectoderm (excluding neural fold) from an open neurula. Note the formation of spots containing chromatophores presumably derived from outside the graft. F. Dorsal surface of a half grown adult which had a portion of dorsal skin rotated 180° at a mid-larval stage. The edges of graft had cut through spot areas leaving deficiencies that are now located 180° away. Note the exactness of fit between the separated spot areas. (From [7]).

is the agent responsible for its manifestation.

The important problem of the mechanism of pigment pattern determination remains unsolved, but some evidence about the temporal aspects of this determination is available. With embryos of *R. pipiens* it was observed that if a portion of the dorsal ectoderm just lateral to, but separate from the neural fold, is transplanted orthotopically to the mid-ventral surface, the transplant differentiates into an island of dorsal integument (Fig. 8B, C, D, E) replete with dorsal pigmentation, skin glands, etc. [7]. Thus, by the open neural plate stage, dorsal ectoderm has been determined as dorsal. When neural crest cells (chromatoblasts) migrate ventrally, those that ultimately reside in programmed ventral ectoderm, behave accordingly and only iridophores differentiate; however, those that invade the dorsal transplant differentiate as they do in dorsal ectoderm and a full range of chromatophore expression is achieved and even a spot pattern is manifested (Fig. 8E). However, the size and conformation of the spots that form in this transplant differ markedly from those on the dorsal surface (Fig. 8A) and lead to the conclusion that at the time of the transplantation, the dorsal ectoderm had been determined to be dorsal, but only generally so, such that the specific details of the dorsal developmental patterns were not yet fixed. Thus, the transplanted dorsal skin regulated (in the embryonic sense) a pattern of its own. Since, a highly specified dorsal pattern is already in place by mid-larval life, it follows that at some period between neurula and mid-larval stages, the already generally determined ectoderm receives a second set of instructions that clearly delineates a highly specific dorsal pattern. Thus, it appears that the two types of adult pigmentation patterns, a dorsal/ventral pattern of dark/light pigmentation, and a specific spot pattern, are separately determined. The mechanisms of these two separate determinations remain unresolved.

Over the years, the cellular basis for the expression of pigment patterns at metamorphosis has been studied on somewhat of a piecemeal basis although the work of Smith-Gill [41, 42, 43] comprises a series of focused labor intensive studies of the morphological changes that occur in the formation of the dorsal pigmentation patterns of the leopard frog. She reported that there was a profound increase in the number of all the dermal chromatophores, but especially the melanophores. This increase is attributed to the differentiation of new melanoblasts and proliferation of existing melanophores. At the same time, a decrease in the number of epidermal melanophores was recorded. The reason for the loss of epidermal melanophores at metamorphosis remained unresolved until relatively recently when Yasutomi [47] discovered that the increase in dermal melanophore number at metamorphosis in *R. japonica* was partially due to the migration of epidermal melanophores to the dermis where they acquire a dermal melanophore morphology. Of particular importance in this study was the demonstration that this migration could be induced in organ culture of dorsal skin in the presence of thyroxine. Thus, it appears that thyroxine also acts directly on the migration of epidermal melanophores and that any indirect effects of thyroxine on MSH release from the pituitary [17] can be precluded. It seems likely that thyroxine acts directly on chromatoblast differentiation or on the proliferation of dermal pigment cells at metamorphosis since it has been shown that the implantation of thyroxine/cholesterol pellets under the skin of *Pachymedusa dacnicolor* tadpoles leads to localized pigment cell changes including the production of mosaic chromatophores possessing both reflecting platelets and pterinosomes [14]. In addition, it has been shown that this hormone directly affects the metabolism of some chromatophore pigments at metamorphosis. In the newt, *Pleurodeles waltlii,* a xanthophore pigment, pleurodeles blue, disappears from the skin abruptly at metamorphosis and its localized disappearance can be induced by the local implantation of thyroxine/cholesterol pellets [3]. The identity of pleurodeles blue remained unresolved for almost 25 years before it was identified to be a novel pyridine derivative [48]. The role of thyroxine in the disappearance of this compound from the skin at metamorphosis is a question of broad implications that begs to be answered.

Other Hormones

The effects of hormones other than MSH and

thyroxine on the physiological control of pigment cells has been known for some time (see [5]); however, it seems that so far only prolactin and the sex steroids are possible candidates as regulators of developmental changes in amphibian pigmentation. Prolactin has long been known to play a major role in bringing about the various changes that occur in second metamorphosis of newts [28]. Among the integumental events that take place are changes in pigmentation; however, there is no clear cut demonstration of a role for prolactin in inducing these changes either directly or indirectly. The case for sex hormones playing a role in the development of amphibian color patterns is more compelling as a result of the studies of Richards [41]. She showed that the administration of estrogen or testosterone to juvenile african reed frogs (*Hyperolius viridiflavus*) could induce the premature expression of the adult bright green and yellow pigmentation pattern of females and some males. Juveniles are normally brown-striped. The rate of change from the juvenile to the adult pattern occurs more rapidly if the steroids are administered during metamorphic climax, and suggests that the actions of the sex hormones are more profound at this period of intense mitosis and pigment cell differentiation. This system seems to be an attractive one for the study of hormonal effects on the developmental biology of pigmentation, but so far it remains unutilized. Another example of a steroid effect on pigmentation that has been under studied concerns the darkening of the dewlap in males of some adult anurans. While this is a well known male secondary sexual characteristic, the possible direct effects of androgens on the ontogenetic expression of this melanization remains to be investigated.

DETERMINATION OF PIGMENT PATTERN *IN SITU*

It seems clear that the two unrelated hormones, MSH and thyroxine are both active in the development of pigmentation, but in both cases their time of action is relatively late and their roles are permissive. As would be expected, pigmentation patterns seem to be determined early in embryonic and larval life; MSH and thyroxine function in the realization of these pre-determined patterns. With the acceptance of this premise comes the need to understand the mechanisms whereby these patterns are actually determined in the embryonic sense. With respect to what was mentioned earlier about the development of spot patterns in *R. pipiens,* a question of concern is, what determines dorsal ectoderm to be "dorsal" by at least the open neural plate stage? This question can not be answered at present; however, it seems likely that the underlying dorsal mesoderm may provide the inductive stimulus for this determination.

Subsequent to the embryonic determination of dorsality and ventrality, in a general sense, comes the second step in determination, that of the high degree of specification revealed by the skin rotation experiments [7]. At this time in larval life, the exact position of spots and interspots is specified and while the exact larval stage at which this occurs has not yet been ascertained, our preliminary unpublished observations indicate that specification has already occurred by stage 4 of Taylor Kollros [44] and that it is probably in place by stage 2 at which time the larvae are only about 20 mm in length. Of course, the mechanism for this second inductive step, the exact specification of pigmentation pattern, is unknown. However, there is good reason to believe that its implementation involves the action of intrinsic highly localized molecules in the integument that either stimulate or inhibit the differentiation of appropriate pigment cells to provide the circumscribed pigmentation patterns [15].

One of the candidate molecules for the implementation of pigment pattern is the melanization inhibiting factor, MIF, which has received much attention since its discovery [26]. This high molecular weight protein (200 to 400 kD) extracted from the ventral skin of several anurans, has the capacity to inhibit melanization of embryonic melanoblasts even in the presence of high levels of MSH [27]. It is thought to be the major factor in maintaining the whitish ventral surface of anurans (and probably other cold-blooded vertebrates) by inhibiting the melanization of latent melanoblasts and stimulating the expression of iridophores [11]. Purification of MIF has been a difficult task, but a partially purified MIF has been used to generate an anti-MIF

monoclonal antibody and its subsequent use as a probe has revealed MIF to be localized in specific regions of the ventral integument of R. pipiens adults (Fukuzawa, unpublished and published in abstract form as [40]).

While investigations on the biological and biochemical characterization of MIF continue, a complementary melanization stimulating factor (MSF) has emerged from studies on the integument of the catfish, *Ictalurus punctatus* [33, 49]. Purification of MSF and its biological and biochemical characterization is in its infant stages; however, MSH activity has been discovered in the skin of other species including *R. pipiens* [36]. An investigation of the spot pattern of this species has revealed that the black spots contain higher MSF activity than do the interspot areas. In the light of these most recent observations, it is attractive to consider that the dorsal spot pattern of *R. pipiens* is expressed because of the localized distribution of MSF molecules in exact accordance with this spot pattern. At the same time, in an extension of this reasoning, the white ventral pattern would result from the appropriate ventral localization of MIF. Whether the putative MSF of *R. pipiens* is chemically related to that of the catfish, the marine teleost, *Sparus auratus* (Zuasti *et al.*, in preparation), or the Long/Evans rat [32] is as yet unknown. However, given the many homologies of pigmentary systems among vertebrates, such a possibility is likely and it may very well be that such a molecule is an important one in the establishment of pigmentation patterns of all vertebrate species.

CONCLUSION

The pigmentation patterns of amphibians are striking and diverse. The development of these patterns is amenable to experimentation and the many studies of this problem have included the possible roles of hormones. Two important hormones, MSH and thyroxine, have been shown to affect the expression of pigment cells during development, but the nature of their respective roles seems permissive rather than causal. The basis for the circumscribed pigmentation patterns seems to be more likely related to intrinsic factors localized in the skin of all vertebrates so far studied. These include a melanization inhibiting factor (MIF) and a melanization stimulating factor (MSF) that are present in adult skin. Their presence in embryos has not been disclosed, but it seems likely that they are present in embryos, for early neural crest cells strikingly respond to putative MIFs or MSFs. It is attractive to consider that the action of these two factors in localized areas of the embryo are responsible for the initiation of pattern formation and that an interplay between these two factors in the presence of an appropriate hormonal ambiance, provides the basic mechanism for the development and establishment of pigment patterns of amphibians and, indeed, all vertebrates.

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